



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> CONJUGATED MUCIN PEPTIDE VACCINES  <b>(57) Abstract</b>  This invention provides a vaccine capable of producing an immune response which recognizes a mucin, comprising an amount of mucin peptide conjugated to an immunogenic protein effective to stimulate or enhance immune response in the subject, an effective amount of an adjuvant and a pharmaceutically acceptable vehicle. This invention further provides a method for stimulating or enhancing production of an immune response which recognizes a mucin, comprising administering to the subject the above-described vaccine.		

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CONJUGATED MUCIN PEPTIDE VACCINES

5 The invention disclosed herein was made with government support under NIH Grant No. CA 61422 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

10 Background of the Invention

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by  
15 reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

20 Mucins, such as the mucin MUC1, are extensively glycosylated high molecular weight (> 200 kD) proteins abundantly expressed in many human cancers of epithelial origin (1-5). Recent studies have demonstrated that MUC1 contains a variable number of tandem repeats of a 20 amino  
25 acid peptide (PDTRPAPGSTAPPAHGVTSA) (SEQ ID. 7) in the extracellular portion of the molecule and that the most antigenic epitome recognized by anti-mucin mAbs and cytotoxic T cells is the APDTR segment within the repeats (6-8). Expression of MUC1 on normal tissues is largely  
30 restricted to the apical surface of secretory cells (1, 4), a site with minimal access to the immune system. In addition, the extensive glycosylation of MUC1 expressed on normal tissues may further limit the immune system exposure to the peptide backbone. It has been suggested that the  
35 peptide backbones of mucins are not fully glycosylated in carcinomas, resulting in exposure to the immune system of peptide sequences which are not normally exposed (6). Consequently, MUC1 peptide specific monoclonal antibodies show specificity for carcinoma-associated mucins from

cancers of breast, pancreatic and ovary origin though the amino acid sequences in both normal and carcinoma mucins are probably the same (9). Immunization of rats with a vaccinia recombinant expressing MUC1 has resulted in protection from challenge with MUC1-expressing tumor cells (5). These findings have suggested that immunization against MUC1 may be possible and that this immunization might prevent tumor regrowth in patients with breast or pancreatic cancer. In this study, applicants' synthesized MUC1 peptides of different lengths and sequences and prepared vaccines containing these peptides mixed with various adjuvants or covalently attached them using different linkers to the protein carrier keyhole limpet hemocyanin (KLH). In preparation for clinical trials, the impact of the different vaccines on humoral and cellular immunological responses and protection from tumor challenge was compared in mice.

Human mucin MUC1 is abundantly expressed in some cancers of epithelial origin and is largely restricted to the apical surface of secretory cells in normal tissues. It is therefore a potential target for cancer immunotherapy. In preparation for clinical trials, vaccines containing synthetic MUC1 peptides of different lengths and sequences mixed with various adjuvants or covalently attached, using different linker methods, to protein carrier keyhole limpet hemocyanin (KLH) were studied in mice. MUC1 peptides (containing 30 amino acids), plus adjuvants QS-21 or BCG, were incapable of inducing antibody. However, MUC1 peptide conjugated to KLH (MUC1-KLH), plus QS-21, induced high titer antibody against the immunizing peptides and against MUC1-expressing tumor cells. Although T cell responses including delayed type hypersensitivity, lymphocyte proliferation and cytotoxic T lymphocyte were not observed in mice immunized with any of these vaccines, significant protection from MUC1-expressing tumor cell challenge in mice immunized with MUC1-KLH was observed. Based on these

studies, a vaccine containing MUC1-KLH conjugate prepared with m-Maleimidobenzoyl-N-hydroxysuccinimide ester linker, plus QS-21, has been constructed for testing in clinical trials.

Summary of the Invention

5 This invention provides a vaccine capable of producing an immune response which recognizes a mucine, comprising an amount of mucin peptide conjugated to an immunogenic protein effective to stimulate or enhance immune response in the subject, an effective amount of an adjuvant and a pharmaceutically acceptable vehicle.

10 In an embodiment, the subject is a human.

In another embodiment, the immunogenic protein is Keyhole Limpet Hemocyanin or a derivative of Keyhole Limpet Hemocyanin.

15 In a separate embodiment, the mucin is MUC1. The mucin may include other mucins such as MUC 2-5. A person of ordinary skill in art would be able to apply this invention in other mucins.

20 In a further embodiment, the MUC1 peptide ranges from ten amino acids to three hundred amino acids in length.

25 In an embodiment, the effective amount of conjugated MUC1 peptide is an amount between about 1  $\mu$ g and about 1mg. In another embodiment, the adjuvant is QS-21.

30 In an embodiment, the effective amount of QS-21 is an amount between about 10 $\mu$ g and about 200 $\mu$ g. In a separate embodiment, the effective amount of QS-21 is about 100  $\mu$ g.

35 In another embodiment, the subject is afflicted with cancer and the immune response produced in the subject upon administration of the vaccine effectively treats the cancer. In a further embodiment, the subject is susceptible to cancer and the immune response produced in the subject upon administration of the vaccine effectively prevents the

cancer. In an embodiment, cells of the cancer have the mucin on their surface.

5 In a further embodiment, the cancer is a breast cancer, prostate cancer, colon cancer, lung cancer or pancreas cancer. This invention is applicable to other cancers of epithelial origin.

10 This invention also provides a method of producing an immune response which recognizes the mucin comprising administering to the subject an effective dose of the above-described vaccine.

15 This invention provides a method for treating cancer in a subject afflicted with cancer comprising administering to the subject an effective dose of the above-described vaccine.

20 This invention provides a method for preventing cancer in a subject susceptible to cancer comprising administering to the subject an effective dose of the above-described vaccine.

25 In the above methods, the immunogenic protein may be Keyhole Limpet Hemocyanin or a derivative of Keyhole Limpet Hemocyanin. In an embodiment of the above methods the adjuvant is QS-21.

Brief Description of the Figures

Figure 1 Conjugation of MUC1 to KLH.



**Detailed Description of the Invention**

This invention provides a vaccine capable of producing an immune response which recognizes a mucin, comprising an amount of mucin peptide conjugated to an immunogenic protein effective to stimulate or enhance immune response in the subject, an effective amount of an adjuvant and a pharmaceutically acceptable vehicle. In an embodiment, the subject is a human.

The effective amount of the conjugated mucin peptide may easily be determined by simple titration experiment. Animals may be immunized with different amounts of the conjugated peptide and tested with the immune response generated. The effective amount will generate an appropriate immune response.

In another embodiment, the immunogenic protein is Keyhole Limpet Hemocyanin or a derivative of Keyhole Limpet Hemocyanin. Other appropriate immunogenic proteins may also be used in this invention. An ordinary skilled artisan may test the appropriateness of an immunogenic protein by conjugating the tested immunogenic protein with a mucin peptide known to be capable of illiciting an immune response. The mucin peptide conjugated may be administrated in animals to test whether it can generate good immune responses. Proteins with good immune response are the appropriate immunogenic proteins.

In a separate embodiment, the mucin is MUC1. The mucin may include other mucins such as MUC 2-5. A person of ordinary skill in the art would be able to apply this invention in other mucins.

In a further embodiment, MUC1 peptide ranges from thirty amino acids to three hundred amino acids in length. In a specific embodiment, the mucin peptide is selected from a

group consisting of APDTRPAPGSTAPPAHGVTS,  
TAPPAHGVTSAPDTRPAPGS, APDTRPAPGSTAPPAHGVTSAPDTRPAPGS,  
VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA, and  
(VTSAPDTRPAPGSTAPPAHG)<sub>2</sub>VTSAPDTRPA. In a further specific  
5 embodiment, the mucin peptide is  
VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA.

In a preferred embodiment, the effective amount of  
conjugated MUC1 peptide is an amount between about 1  $\mu$ g and  
10 about 1mg.

In another embodiment, the adjuvant is QS-21. As it can be  
readily appreciated by persons of ordinary skill in the  
art, other appropriate adjuvants may be similarly used.  
15

In a preferred embodiment, the effective amount of QS-21 is  
an amount between about 10 $\mu$ g and about 200 $\mu$ g. In a further  
preferred separate embodiment, the effective amount of QS-  
21 is about 100  $\mu$ g.  
20

In another embodiment, the subject is afflicted with cancer  
and the immune response produced in the subject upon  
administration of the vaccine effectively treats the  
cancer.  
25

In a further embodiment, the subject is susceptible to  
cancer and the immune response produced in the subject upon  
administration of the vaccine effectively prevents the  
cancer. In an embodiment, cells of the cancer have the  
30 mucin on their surface.

In a further embodiment, the cancer is a breast cancer,  
prostate cancer, colon cancer, lung cancer or pancreas  
cancer. This invention is applicable to other cancers of  
35 epithelial origin.

This invention also provides a method for stimulating or

enhancing in a subject production of an immune response which recognizes the mucin comprising administering to the subject an effective dose of the above-described vaccine.

5 This invention provides a method for treating cancer in a subject afflicted with cancer comprising administering to the subject an effective dose of the above-described vaccine.

10 This invention provides a method for preventing cancer in a subject susceptible to cancer comprising administering to the subject an effective dose of the above-described vaccine.

15 In the above methods, the immunogenic protein includes, but is not limited to Keyhole Limpet Hemocyanin or a derivative of Keyhole Limpet Hemocyanin.

20 In an embodiment of the above methods, the adjuvant is QS-21.

In a separate embodiment, the mucin is MUC1. The mucin may include other mucins such as MUC 2-5. A person of ordinary skill in the art would be able to apply this invention in other mucins.

25 The mucin may include other mucins such as MUC 2-5. A person of ordinary skill in art would be able to apply this invention in other mucins.

30 In a further embodiment, MUC1 peptide ranges from thirty amino acids to three hundred amino acids in length. In a specific embodiment, the mucin peptide is selected from a group consisting of APDTRPAPGSTAPPAHGVTS, TAPPAHGVTSAPDTRPAPGS, APDTRPAPGSTAPPAHGVTSAPDTRPAPGS, VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA, and (VTSAPDTRPAPGSTAPPAH)<sub>2</sub>VTSAPDTRPA. In a further specific

35

embodiment, the mucin peptide is  
VTSAPDTRPAPGSTAPPAGVTSAPDTRPA.

5 In a preferred embodiment, the effective amount of  
conjugated MUC1 peptide is an amount between about 1  $\mu$ g and  
about 1mg.

10 In another embodiment, the adjuvant is QS-21. As it can be  
readily appreciated by persons of ordinary skill in the  
art, other appropriate adjuvants may be similarly used.

15 In a preferred embodiment, the effective amount of QS-21 is  
an amount between about 10 $\mu$ g and about 200 $\mu$ g. In a further  
preferred separate embodiment, the effective amount of QS-  
21 is about 100  $\mu$ g.

In an embodiment, cells of the cancer have the mucin on  
their surface.

20 In a further embodiment, the cancer is a breast cancer,  
prostate cancer, colon cancer, lung cancer or pancreas  
cancer. This invention is applicable to other cancers of  
epithelial origin.

25 This invention will be better understood from the  
Experimental Details which follow. However, one skilled in  
the art will readily appreciate that the specific methods  
and results discussed are merely illustrative of the  
invention as described more fully in the claims which  
30 follow thereafter.

Experimental DetailsFIRST SERIES OF EXPERIMENTS

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## MATERIALS AND METHODS

Materials. Peptides. MUC1 peptides containing 20, 30 and 50 amino acids with different sequences were synthesized using an Applied Biosystems Model 431A automated peptide synthesizer in the Core Facilities of Memorial Sloan-Kettering Cancer Center. A cysteine was added as indicated to the original sequence at the C- or N-terminal of the synthetic peptides to facilitate conjugation with protein carriers (Table 1).

Table 1 Sequences of Synthetic MUC1 Peptides Used For Vaccine Preparation and Testing

20	Peptide	Amino Acid Sequence
	MUC1(20A)	APDTRPAPGSTAPPAHGVTS(C) (SQ ID 2)
	MUC1(20)	TAPPAHGVTSAPDTRPAPGS(C) (SQ ID 3)
25	MUC1(30A)	APDTRPAPGSTAPPAHGVTSAPDTRPAPGS(C) (SQ ID 4)
	MUC1(30)	(C)VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA (SQ ID 5)
30	MUC1(50)	(C)(VTSAPDTRPAPGSTAPPAHG) <sub>2</sub> VTSAPDTRPA (SQ ID 6)

MAbs and cell lines. HMFG-2 is a MUC1-reactive mouseIgG mAb (10). 410.4 is a murine (BALB/c) mammary epithelial cancer cell line (11) and E4 is derived from a clone of 410.4 cells transfected with the MUC1 gene (12). HMFG-2, 410.4 and E4 were kindly provided by Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London, U.K.). MCF 7 is a human breast carcinoma cell line (13).

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Animals and adjuvants. Female BALB/c x C57BL/6 F1 mice,

BALB/c x C3H F1 mice , or BALB/c mice, 6 weeks of age, were obtained from the Jackson Laboratory (Bar Harbor, Maine). Adjuvant QS-21, a purified saponin fraction (16), was obtained from Cambridge Biotech, Inc. (Worcester, MA).  
5 Bacille Calmette-Guerin (BCG) was purchased from Connaught Laboratories (Ontario, Canada).

Conjugation of MUC1 peptides to keyhole limpet hemocyanin (KLH). KLH (PerImmune Inc., Rockville, MD) was used as  
10 carrier protein for MUC1 peptide conjugates. m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, Pierce Co., Rockford, IL), N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Pierce Co., Rockford, IL) and glutaraldehyde (Aldrich Chemical Co., Milwaukee, WI) were  
15 used as linkers for making MUC1 peptide conjugates (Fig.1).

MBS conjugation method (14). One mg MBS in 70 $\mu$ l dimethylformamide (Sigma Chemical Co., St. Louis, MO) was added to 5 mg KLH in 1 ml 0.01 M phosphate buffer (PB) pH  
20 7.0. One hour later, MBS/KLH solution was applied on Sephadex G-15 column equilibrated with 0.1 M PB pH 6.0. The first peak at OD280 (MBS-KLH) was collected and mixed with 5 mg MUC1 peptide and stirred at room temperature for 2 hours. The unconjugated peptide was separated from MUC1-KLH  
25 conjugate using a Centriprep-30 concentrator (Amicon Inc., Beverly, MA). The MUC1/KLH conjugation ratio (500/1 - 600/1) was calculated based on the starting amount of peptide and KLH and the amount of unconjugated peptide in the filtrate by spectrophotometer.

30 SPDP conjugation method (15). Conjugation by the SPDP method was similar to that by the MBS method except that MBS was replaced by SPDP. The conjugate ratio of MUC1/KLH resulting from the SPDP method was calculated in 2  
35 different assays: 1) based on the amount of unconjugated peptide, and 2) based on the SPDP by-product (pyridine-2-thione) produced by the conjugation reaction. The MUC1/KLH

ratio was 400/1 -500/1 by both assays.

5 Glutaraldehyde conjugation method (14). Five mg KLH in 1 ml borate buffer pH 10 was mixed with 5 mg MUC1 peptide. One ml of 0.3% glutaraldehyde was added and stirred at room temperature for 2 hours. Unreacted glutaraldehyde was blocked by adding 0.25 ml 1 M glycine for 30 min. The MUC1-KLH solution was dialysed against PBS overnight. Because glutaraldehyde interfered with the absorbance of unconjugated peptide at OD215, the ratio of MUC1/KLH was assumed based on the starting ratio, 500/1.

15 Vaccine preparation and administration. Mice were immunized with 8-15  $\mu$ g MUC1 peptide alone or conjugated to KLH plus 8-10  $\mu$ g QS-21 or  $5 \times 10^5$  BCG, 2-3 times at one week intervals. Eight to ten days after the 2nd or 3rd immunization, mice were bled and the sera separated for testing with ELISA and flow cytometry assays.

20 Serological assays. ELISA. ELISAs were performed as previously described (17). MUC1 peptide in 0.1 M carbonate buffer pH 9.6 were coated on ELISA plates at 0.1  $\mu$ g/well. A series of antiserum dilutions were incubated with the coated peptide for 1 hour. Secondary antibodies were 25 alkaline phosphatase-conjugated goat anti-mouse IgG or IgM at a dilution of 1/200 (Southern Biotechnology Associates, Inc., Birmingham, AL). ELISA titer is defined as the highest dilution yielding an absorbance of 0.1 or greater over that of normal mouse sera. MAb HMFG-2 was used as 30 positive control in each assay.

Flow cytometry. Tumor cells ( $2 \times 10^5$ ) were incubated with 40 l of 1/30 diluted antisera or 1/2 diluted mAb supernatant for 30 min on ice. After washing with 3% fetal calf serum/phosphate buffered saline, the cells were incubated 35 with 20  $\mu$ l of 1/15 diluted fluorescein-isothiocyanate-labeled goat anti-mouse IgM or IgG (Southern Biotechnology

Associates, Inc., Birmingham, AL). The positive population of the stained cells were quantitated by flow cytometry (EPICS-Profile II, Coulter Co., Hialeah, FL), as previously described (18).

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T lymphocyte assays. Proliferative assay (19). Lymphocytes ( $2 \times 10^5$ /well) were prepared from the spleens of mice seven days after the 2nd immunization, and incubated with MUC1 peptide (0.1-10  $\mu$ g/ml) or KLH (1-20  $\mu$ g/ml) in 37°C/5% CO<sub>2</sub> for 3-5 days. Eighteen hours after adding 0.5  $\mu$ Ci <sup>3</sup>H-Thymidine (ICN, Irvine, CA) per well, the cells were processed and analyzed with a 1204 Betaplate (Wallac Oy, Finland).

15 Delayed type hypersensitivity (DTH) (20). Two weeks after the 2nd immunization, 5  $\mu$ g MUC1 peptide or KLH were injected in 20  $\mu$ l PBS into the hind foodpad. Footpad thickness was measured at 24 and 48 hours.

20 Cytotoxic T lymphocyte (CTL) assay (21). Seven days after the 2nd immunization, splenic lymphocytes were sensitized in vitro with 1-8  $\mu$ g/ml MUC1 peptide and 10 unit/ml IL-2 (Boehringer Mannheim, Germany) for 7-10 days. The sensitized lymphocytes were then incubated with europium-labeled E4 or 410.4 cells at ratios of 10:1-100:1 for 4  
25 hours. Percent release of europium from target cells were measured with a time-resolved 1232 Delfia fluorometer (Wallac Oy, Finland) (18).

30 Tumor challenge in mice. Three days after the 2nd immunization, mice were injected i.v. with  $2 \times 10^5$  E4 cells. Twenty five days later, the mice were sacrificed, the lungs fixed with 10% formaldehyde, and the number of tumor colonies in the lungs were counted, as previously described  
35 (22).



## EXPERIMENTAL RESULTS

Effect of MUC1-KLH Vaccine Construction on Serological Response. Comparison of MUC1 peptides and MUC1 conjugates (Table 2). After 3 vaccinations, sera from 6 mice immunized with 15  $\mu$ g peptide MUC1(30) plus 10  $\mu$ g QS-21 or  $5 \times 10^5$  BCG per vaccination failed to show significant titers of IgM or IgG antibody against MUC1, while sera from mice immunized with 15  $\mu$ g MUC1(30) conjugated with KLH plus 10  $\mu$ g QS-21 showed high titer IgG and modest titer IgM (median titer 1:409600 and 1:800, respectively). Sera from mice immunized with MUC1(30)-KLH, but not those immunized with unconjugated MUC1, also showed strong reactivity with MUC1-expressing E4 murine breast cancer cells and MCF 7 human breast cancer cells.

Table 2. Serological Response with Antisera from Mice Immunized with MUC1 Peptide or MUC1-KLH Conjugates<sup>a</sup>

		ELISA Titers On Immunizing MUC1 peptide	Flow Cytometry (IgG) $\pm$ positive cells	
	Antisera	IgM	IgG	E4 cells    MCF7 cells
	<u>Controls</u>			
	unprimed mice 1	blank <sup>d</sup>	blank	4            5
	unprimed mice 2	blank	blank	2            4
	anti-GD3-KLH 1	< 1:20	< 1:25	2            5
	anti-GD3-KLH 2	< 1:20	< 1:25	4            7
	mAb HMFG-2	-	1:320	94           57
	<u>Muc1(30) + BCG</u>			
	G2-1	< 1:20	< 1:25	3            -
	G2-2	< 1:20	< 1:25	2            -
	G2-3	< 1:20	< 1:25	4            -
	G2-4	< 1:20	< 1:25	3            -
	G2-5	< 1:20	< 1:25	1            -
	G2-6	< 1:20	< 1:25	1            -
	<u>median</u>	<u>&lt; 1:20</u>	<u>&lt; 1:25</u>	<u>3</u> <u>4</u> <sup>b</sup>
	<u>Muc1(30) + QS21</u>			
	G3-1	< 1:20	< 1:25	1            -

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	G3-2	< 1:20	< 1:25	1	-
	G3-3	< 1:20	< 1:25	1	-
	G3-4	< 1:20	< 1:25	1	-
	G3-5	< 1:20	< 1:25	1	-
5	G3-6	< 1:20	< 1:25	1	-
	<b><u>median</u></b>	<b><u>&lt; 1:20</u></b>	<b><u>&lt; 1:25</u></b>	<b><u>1</u></b>	<b><u>3<sup>b</sup></u></b>
	Muc1(30)-KLH +				
	QS21				
10	G5-1	1:400	1:819200	93	69
	G5-2	1:800	1:204800	66	25
	G5-3	1:800	1:819200	86	56
	G5-4	1:1600	1:819200	97	79
	G5-5	1:800	1:204800	72	40
15	G5-6	1:3200	1:204800	94	67
	<b><u>median</u></b>	<b><u>1:800</u></b>	<b><u>1:409600</u></b>	<b><u>90</u></b>	<b><u>62</u></b>
	<u>Muc1(30A)-KLH +</u>				
	<u>QS21</u>				
20	G4-1	1:800	1:409600	83	83
	G4-2	1:800	1:819200	92	58
	G4-3	1:800	1:204800	95	45
	G4-4	1:400	1:204800	81	20
	G4-5	1:1600	1:819200	93	57
25	G4-6	1:400	1:204800	85	48
	<b><u>median</u></b>	<b><u>1:800</u></b>	<b><u>1:307200</u></b>	<b><u>89</u></b>	<b><u>53</u></b>
	<u>Muc1(50)-KLH +</u>				
	<u>QS21</u>				
30	G6-1	1:3200	1:204800	73	40
	G6-2	1:3200	1:204800	89	52
	G6-3	1:3200	1:409600	98	79
	G6-4	1:3200	1:409600	86	57
	G6-5	1:1600	1:409600	97	72
35	G6-6	1:3200	1:409600	83	53
	<b><u>median</u></b>	<b><u>1:3200<sup>c</sup></u></b>	<b><u>1:409600</u></b>	<b><u>88</u></b>	<b><u>55</u></b>

<sup>a</sup> All the MUC1 conjugates in this table were made by the MBS method. Mice immunized with 15 g MUC1-KLH /mouse or MUC1 peptide 15 g/mouse plus QS-21 10 g/mouse or BCG 3 x 10<sup>5</sup>/mouse were bled 7 days after the 3rd vaccines.

<sup>b</sup> Test value obtained from pooled sera from all 6 mice in this group.

<sup>c</sup> Compared with MUC1(30A)-KLH and MUC1(30)-KLH groups, p < 0.01 and p < 0.05 by Mann-Whitney/Wilcoxon non-parametric statistics.

<sup>d</sup> Absorbance values of the sera from unprimed mice were used as background values for subtraction.

5 Comparison of conjugation methods (Table 3). The median IgG  
titer of sera from mice immunized with MUC1(20)-KLH  
conjugated using glutaraldehyde and MUC1(20)-KLH and  
MUC1(30A)-KLH conjugated using SPDP were 1:1350, 1:50 and  
1:2700, respectively. The median IgG titer after  
10 immunization with MUC1(20)-KLH and MUC1(30A)-KLH conjugated  
using MBS were 1:12150 and 1:12150 respectively,  
significantly higher than those prepared using  
glutaraldehyde or SPDP ( $p < 0.01$ ). Likewise, the median %  
positive cells by flow cytometry with sera from mice  
15 immunized with MUC1(20)-KLH (glutaraldehyde), MUC1(20)-  
KLH (SPDP) and MUC1(30A)-KLH (SPDP) were 59%, 32% and 61%  
respectively. Both the MUC1(20)-KLH (MBS) and MUC1(30A)-KLH  
(MBS) groups had 97% positive cells, significantly higher  
than those conjugates prepared by glutaraldehyde or SPDP ( $p$   
20  $< 0.05$ ). For MUC1(20A) conjugates, on the other hand, no  
obvious difference in titer or % positive cell were  
observed between the MBS and SPDP methods. Reactivity  
against the MUC1-negative cell line 410.4 was minimal in  
all groups.

25 Comparison of different length MUC1 peptides (Table 3).  
Median IgG titers against the immunizing peptides were  
similar for sera from mice immunized with MUC1(20A)-KLH  
(1:4500 (SPDP) and 1:4500 (MBS)) and MUC1(30A)-KLH (1:2700  
30 (SPDP) and 1:12150 (MBS)). Although the median % positive  
cells for MUC1(30A)-KLH (SPDP) (61%) was slightly but not  
significantly higher than for MUC1(20A)-KLH (SPDP) (32%),  
the median % positive cells for MUC1(30A)-KLH (MBS) (97%)  
was significantly higher than for MUC1(20A)-KLH (MBS)  
35 (54%). When MUC1(50)-KLH was compared with MUC1(30)-KLH and  
MUC1(20)-KLH (Tables 2 and 3), no significant difference in  
% positive cells was found.

Table 3. Serological Response with Antisera from Mice Immunized with MUC1 conjugated to KLH by Different Methods<sup>a</sup>

		ELISA peptide (IgG)	Flow Cytometry (IgG)	
			410.4 cell	E4 cell
5	Mixed normal	blank	4	2
	mAb HMFG II	1:320	1	99
	Glutaraldehyde			
	MUC1 (20) -KLH			
10	1	4050	4	77
	2	1350	14	53
	3	1350	9	59
	<u>median</u>	<u>1350</u>	<u>9</u>	<u>59</u>
	SPDP method			
15	MUC1 (20A) -KLH			
	1	4050	4	4
	2	4050	14	32
	3	4050	10	91
	<u>median</u>	<u>4050</u>	<u>10</u>	<u>32</u>
20	MUC1 (20) -KLH			
	1	1350	5	96
	2	50	27	43
	3	50	7	16
	4	50	2	55
25	5	150	3	2
	6	50	11	21
	<u>median</u>	<u>50</u>	<u>6</u>	<u>32</u>
	MUC1 (30A) -KLH			
	1	4050	11	85
30	2	4050	10	96
	3	4050	4	56
	4	150	11	44
	5	450	2	30
	6	1350	3	65
35	<u>median</u>	<u>2700</u>	<u>7</u>	<u>61</u>
	MBS method			
	MUC (20A) -KLH			
	1	4050	11	61
	2	4050	6	54
40	3	12150	8	54
	<u>median</u>	<u>4050</u>	<u>8</u>	<u>54</u>
	MUC1 (20) -KLH			
	1	12150	6	89
	2	12150	14	99
45	3	12150	8	95
	4	36450	6	99
	5	109350	5	99
	6	12150	10	92
	<u>median</u>	<u>12150<sup>b</sup></u>	<u>7</u>	<u>97<sup>b</sup></u>
50	MUC1 (30A) -KLH			
	1	12150	8	95
	2	12150	6	99
	3	12150	12	86
	4	12150	3	98
55	5	12150	9	60
	6	36450	3	99 <sup>b</sup>
	<u>median</u>	<u>12150<sup>b</sup></u>	<u>7</u>	<u>97</u>

<sup>a</sup> Ten days after the 3rd immunization with 10 g MUC1-KLH/mouse plus QS-21 10 g/mouse. Number 1-3 of mice in each group are strain CB6F1 while number 4-6 are BALB/c.

5 <sup>b</sup> Compared with the corresponding MUC1-KLH conjugated by SPDP or Glutaradehyde method, using Mann-Whitney/Wilcoxon non-parametric statistics,  $p < 0.01$  for ELISA titer,  $p < 0.05$  for % positive cells.

10 Serological response to conjugated MUC1 with different sequences (Table 3). The median IgG titer against the immunizing peptides of sera from mice immunized with MUC1(20)-KLH (SPDP) (1:50) was obviously lower than that from mice immunized with MUC1(20A)-KLH (SPDP) (1:4050).  
15 However, the median % positive cells by flow cytometry with sera from these two groups was the same (32%). Both median IgG titer (1:12150) and % positive cells (97%) from mice immunized with MUC1(20)-KLH (MBS) were significantly higher than that for mice immunized with MUC1(20A)-KLH (MBS),  
20 1:4050 and 54% respectively ( $p < 0.05$ ). When MUC1(30)-KLH was compared with MUC1(30A)-KLH (Table 2), no obvious difference in serological response was found.

Effect of MUC1 immunization on T cell responses. All the  
25 mice in Tables 3 and 4 were tested for DTH. Forty eight hours after footpad injection with 5  $\mu$ g MUC1 peptide, the average footpad thickness for mice immunized with MUC1 peptides and MUC1-KLH constructs were 1.72  $\pm$  0.05 mm (n=12) and 1.73  $\pm$  0.06 mm (n=28) respectively and not  
30 different from 1.73  $\pm$  0.06 mm (n=20) of mice injected with PBS alone. After DTH testing with 5 g KLH (positive control), however, the average footpad thickness for mice immunized with MUC1-KLH was 2.1  $\pm$  0.17 mm (n=8), significantly increased above the KLH DTH response for  
35 unprimed mice and mice immunized with MUC1 peptides, 1.71  $\pm$  0.02 mm (n=6) and 1.71  $\pm$  0.03 mm (n=6) respectively.

The results of proliferative assays were similar to that of DTH assays. Spleen cells from 5 mice for each group  
40 immunized with MUC1(20)-KLH, MUC1(30)-KLH and MUC1(50)-KLH

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or the corresponding peptide did not show increased lymphocyte proliferation above unprimed mice (4530cpm) when these cells were incubated with the corresponding peptide. Increased proliferation (14500-18600 cpm) was observed only when the spleen cells from mice immunized with MUC1-KLH conjugates were incubated with KLH.

An average of 30% specific release in 3 CTL assays on E4 cells (E:T, 60:1) was observed when the effector cells were obtained from BALB/c x C3H F1 mice immunized with mitomycin-treated E4 cells, but no significant specific release (0-2%) were seen from the mice immunized with MUC1 peptides or MUC1-KLH conjugates.

Effect of Active Immunization with MUC1-KLH on E4 cell Lung Metastasis in BALB/c x C3H Mice (Table 4). Ten mice immunized with MUC1(30)-KLH produced IgG antibody at a mean titer of 1:5940 with good E4 cell surface reactivity (mean % positive cells, 52%). Four weeks after i.v. challenge with E4 cells, the mean number of lung colonies from 10 mice was 19 and 20 for PBS and KLH control groups respectively, while only 1 for the MUC1(30A)-KLH group ( $p < 0.01$ ).

Table 4. The Effect of Active Immunization with MUC1(30)-KLH Plus QS-21 on E4 Cell Lung Metastasis<sup>a</sup>

Sera or mAb	ELISA reciprocal titer	Flow Cytometry % positive cells (IgG)		No. of colonies in lungs
		(IgG)	410.4 cell E4 cell	
mAb HMFG-2	320	1	81	
PBS Control				
1-1	-	-	-	44
1-2	-	-	-	51
1-3	-	-	-	6
1-4	-	-	-	1
1-5	-	-	-	5

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	1-6	-	-	-	39
	1-7	-	-	-	2
	1-8	-	-	-	30
	1-9	-	-	-	3
5	1-10	-	-	-	4
	<u>mean<sup>b</sup></u>	<u>blank</u>	<u>5</u>	<u>5</u>	<u>19</u>
	KLH Control				
	2-1	-	-	-	51
	2-2	-	-	-	8
10	2-3	-	-	-	14
	2-4	-	-	-	5
	2-5	-	-	-	48
	2-6	-	-	-	17
	2-7	-	-	-	8
15	2-8	-	-	-	11
	2-9	-	-	-	23
	2-10	-	-	-	14
	<u>mean<sup>b</sup></u>	<u>0</u>	<u>5</u>	<u>5</u>	<u>20</u>
	MUC1(30A) -				
20	KLH				
	3-1	8100	5	50	4
	3-2	8100	5	62	5
	3-3	2700	6	21	3
	3-4	2700	4	84	1
25	3-5	2700	5	26	0
	3-6	2700	5	49	0
	3-7	8100	6	35	0
	3-8	8100	10	63	1
	3-9	8100	5	84	0
30	3-10	8100	6	41	0
	<u>mean</u>	<u>5940</u>	<u>6</u>	<u>52</u>	<u>1<sup>c</sup></u>

<sup>a</sup> BALB/c x C3H F1 were immunized twice at one week interval with 8 g MUC1(30A)-KLH plus 8 g QS-21 /mouse and boosted one week after the 2nd vaccine with 8 g MUC1(30A) peptide plus 8 g QS-21. KLH group were given the same amount of KLH as the MUC1(30A)-KLH group. Three days after 2nd vaccine,  $2 \times 10^5$  E4 cells were injected i.v. to all the groups. Twenty eight days after E4 cell injection, mice were sacrificed to check the metastatic colonies in lungs. Mice were bled 10 days after 2nd vaccine for ELISA and flow cytometry assay.

<sup>b</sup> The test values of ELISA and flow cytometry were obtained from a pool of sera from 10 mice in this group.

<sup>c</sup> Compared with PBS and KLH control groups,  $p < 0.01$ , by Mann-Whitney/Wilcoxon non-parametric statistics.

## EXPERIMENTAL DISCUSSION

MUC1 specific antibodies have been detected in sera from occasional breast, pancreatic and colon cancer patients (2, 23). This suggests that MUC1 can be recognized by the human immune system, and raised the possibility that immunity against tumor cells expressing MUC1 might be induced by properly constructed MUC1 vaccines. Applicants' explored here vaccines containing synthetic MUC1 peptides of different lengths and sequences mixed with various adjuvants or covalently attached using different linker methods to KLH. Vaccines containing unconjugated MUC1 peptides plus QS-21 or BCG failed to induce antibody. However, when the MUC1 peptides were conjugated to KLH, plus QS-21, high titer IgM and, especially, IgG antibodies against MUC1 antigen were successfully induced. The reactivity of these antisera with MUC1-expressing mouse tumor E4 and human breast tumor MCF7 cells was strong, similar to mAb HMFG-2. When conjugation methods utilizing MBS, SPDP and glutaraldehyde were compared, MBS was found to be the best linker for preparing MUC1-KLH conjugates, inducing the most favorable antibody in both titer and specificity. Although conclusions on the effect of MUC1 peptide length on immune response can not be drawn at this time, 30 or 50 amino acid MUC1 conjugates seemed to induce antisera with higher titer against MUC1 positive tumor cells than 20 amino acid MUC1 conjugates. For MUC1 peptide with a single tandem repeat (20 amino acids), APDTR within the peptide, MUC1(20), induced antibody with stronger reactivity against MUC1-expressing E4 cells than MUC1(20A) which contains APDTR at the N-terminal of the peptide. This effect was lost, however, when longer peptides (30 amino acids) were tested.

T lymphocyte immunity against MUC1 peptide in unimmunized cancer patients has been documented (24, 25). In this study, applicants' tested T cell responses in mice



immunized with synthetic MUC1 peptides conjugated to KLH plus QS-21. QS-21 is an immunological adjuvant known to be capable of inducing CTL against other soluble protein antigens (29). However, applicants' did not observe T cell responses (DTH, lymphocyte proliferation or CTL) to MUC1 antigen or MUC1 positive cells after immunization. Others have attempted to induce T cell responses with synthetic MUC1 peptide, MUC1 conjugates, vaccinia expressing MUC1 or MUC1 expressed on whole tumor cells (5, 26-28). By immunizing mice with MUC1(20 amino acids) coupled to diphtheria-toxoid or fused with glutathione-S-transferase, or a natural mucin (human milk fat globule) plus complete Freund's adjuvant, Apostolopoulos et al (26) also failed to induce T cell responses in mice. However, T cell responses were observed after immunizing mice with whole tumor cells expressing MUC1 antigen, as applicants' were also able to demonstrate. DTH but not in vitro immune responses in mice immunized with MUC1(16 amino acids)-KLH were reported by Ding et al (27). These different observations may be due to the use of different strains of mice. While failing to induce T cell responses in mice with MUC1 peptide corresponding to one tandem repeat (20aa), Finn et al found MUC1 peptide corresponding to five tandem repeats has the capacity for CTL induction (30). Whether longer MUC1 peptides, conjugated to KLH or not, also have this capacity is the focus of further study. Since amino acid sequences of natural mucins and motifs of peptides associated with Class I MHC molecules are different in mice than humans (6, 31), T cell responses to MUC1 antigens in humans may be significantly different than those seen in mice. The CTL against MUC1 already identified in unimmunized patients with breast, ovary and pancreatic cancers suggest that this is so (24, 25).

Despite the lack of detected T cell immunity to MUC1 after immunization, protection from challenge with MUC1 positive E4 cells was seen. The number of lung metastases in mice

immunized with MUC(30)-KLH plus QS-21 was significantly lower than that for the PBS or KLH plus QS-21 control groups. This suggests that humoral immunity may be responsible for this resistance to challenge with MUC1-expressing E4 tumor cells. Others have also described protection from challenge with MUC1 positive tumor cells in rodents after immunization. Significant rejection of mucin-expressing tumor occurred in Fisher rats immunized with a vaccinia recombinant expressing MUC1 (5), and prolonged survival of CAF1 mice immunized with MUC1 conjugate BP-1-7-KLH (GVTSAPDTRPAPGSTA) (SQ ID. 1) was described after challenge with MUC1-expressing E3 cells (27). While DTH responses were seen in the BP-1-7-KLH immunized mice, CTL responses were not described. Consequently it is difficult to know from the results in rodent models which arm of the immune system is primarily responsible for protection from tumor challenge, and so it is difficult to predict which assays of immunity will be most important to follow in the clinic in Phase I/II trials designed for optimizing the immunogenicity of MUC1 vaccines.

CTL and antibody responses against MUC1 have been described in unimmunized patients with breast, ovary or pancreatic cancer (2, 23-25). It remains to be determined whether immunity against MUC1 in patients can be augmented by treatment with tumor vaccines. To date only one trial has been conducted, immunization of advanced disease breast cancer patients with a 105 amino acid MUC1 peptide (5 tandem repeats) mixed with BCG (30). While the final results are not available, it appears that neither anti MUC1 antibodies nor DTH were consistently augmented. There was, however, a 2-3 fold augmentation of HLA unrestricted anti- MUC1 CTL precursors in post-immunization blood compared to pre-immunization blood (O. J. Finn, personal communication). However, these results in patients with advanced disease may not be applicable to an adjuvant setting. Our results demonstrate that MUC1-KLH conjugates

are far more potent than MUC1 plus BCG for inducing IgM and IgG antibodies against MUC1. Although T cell responses to MUC1 antigen were not observed in our study, antibodies with high titer against synthetic MUC1 and MUC1-expressing murine and human tumor cells were successfully induced in all mice and this was associated with significant protection from challenge with MUC1-expressing E4 tumor cells. Based on this, applicants' have initiated a clinical trial with MUC1-KLH (30 amino acid) prepared using an MBS linker plus QS-21 and have plans to test a 90 amino acid MUC1 peptide in this setting as well. Assays for humoral, DTH, proliferative and CTL responses will be followed.

Human mucin MUCI is abundantly expressed in many cancers of epithelial origin and is largely restricted to the apical surface of secretory cells in normal tissues. It is therefore a potential target for cancer immunotherapy. In preparation for clinical trials, vaccines containing synthetic MUCI peptides of different lengths and sequences, and mixed with various adjuvants or covalently attached (using different linker methods) to protein carrier keyhole limpet hemocyanin (KLH) were studied in mice. MUCI peptides (containing 20 or 30 amino acids) plus adjuvants QS-21 or BCG did not induce antibody. However, MUCI peptides conjugated to KLH (MUCI-KLH), especially by linker m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), plus QS-21, induced high titer antibody against the immunizing peptides, median titer 1:800 for IgM and 1:307,200 for IgG. In addition, these antisera showed strong reactivity with MUC1-expressing mouse tumor E4 cells and human breast MCF-7 cells, similar to the MUC1- reactive mAb HMFG-2. Based on these studies, a vaccine containing MUC1-KLH conjugate prepared with linker MBS, plus QS-21, has been constructed for testing in clinical trials.

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**References**

1. Arklie, J., Taylor-Paradimitriou, J., Bodmer, W., Egan, M., and Millis, R. Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancers. *Int. J. Cancer*, 28:23-29, 1981.
2. Kotera Y., Fontenot, J. D., Pecher, G., Metzgar, R. S., and Finn, O. J. Human immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic and colon cancer patients. *Cancer Res.*, 54:2856-2860, 1994.
3. Devine, P. L., Layton, G. T., Clark, B. A., Birrell, G. W., Ward, B. G., Xing, P. X., and McKenzie, F.C. Production of MUC1 and MUC2 mucins by human tumor cell lines. *Biochem. Biophys. Res. Commun.*, 178:593-599, 1991.
4. Hollingsworth, M. A., Strawhecker, J. M., Caffrey T. C., and Mack, D. R. Expression of MUC1, MUC2, MUC3 and MUC4 mucin mRNAs in human pancreatic and intestinal tumor cell lines. *Int. J. Cancer*, 57:198-203, 1994.
5. Hareuveni, M., Gautier, C., Kieny, M.-P., Wreschner, D., Chambon, P., and Lathe, R. Vaccination against tumor cells expressing breast cancer epithelial tumor antigen. *Proc. Natl. Acad. Sci. USA.*, 87:9498-9502, 1990.
6. Gendler, S. J., Spicer, A. P., Lalani, E.-N., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Boshell, M., and Taylor-Papadimitriou, J. Structure and biology of a carcinoma-associated mucin, MUC1. *Am. Rev. Respir. Dis.*, 144:S42-S47, 1991.
7. Burchell, J., Taylor-Papadimitriou, J., Boshell, M., Gendler, S., and Duhig, T. A short sequence, within the

amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. *Int. J. Cancer*, 44:691-696, 1989.

- 5        8. Fontenot, J. D., Tjandra, N., Bu, D., Ho, C., Montelaro, R. C., and Finn, O.J. Biophysical characterization of one-, two-, and three-tandem repeats of human mucin (muc-1) protein core. *Cancer Res.*, 53:5386-5394, 1993.
- 10       9. Perez, L., Hayes, D. F., Maimonis, P., Abe, M., O'Hara, C., and Kufe, D. W. Tumor selective reactivity of a monoclonal antibody prepared against a recombinant peptide derived from the DF3 human breast carcinoma-associated antigen. *Cancer Res.*, 52:2563-2568, 1992.
- 15       10. Taylor-Papadimitriou, J., Peterson, J. A., Arklie, J., Burchell, J., Ceriani, R. L., and Bodmer, W. F. Monoclonal antibodies to epithelium specific components of the milk fat globule membrane: production and reactions with cells in culture. *Int. J. Cancer*, 28:17-21, 1981.
- 20       11. Miller, F. R., Miller, B. E., and Heppner, G. H. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. *Invasion Metastasis*, 3:22-31, 1983.
- 25       12. Lalani, E.-N., Berdichevsky, F., Boshell, M., Shearer, M., Wilson D., Stauss, H., Gendler, S. J., and Taylor-Papadimitriou, J. Expression of the gene coding for a human mucin in mouse mammary tumor cells can affect their tumorigenicity. *J. Biol. Chem.*, 266:15420-15426, 1991.
- 30       13. Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, M. A human cell line from a pleural effusion derived from a breast carcinoma. *J. Natl. Cancer Inst.* 51:1409-1416, 1973.
- 35

14. Maloy, W. L., Coligan, J.E., and Paterson, Y. Production of antipeptide antisera. In Current Protocols in Immunology (J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds.) pp. 9.4.1-9.4.11. John Wiley & Sons, Inc. New York, 1994.
15. Carlessen, J., Drevin, H., and Axen, R. Protein thiolation and reversible protein-protein conjugation N-succinimidyl 3-(2-pyridyldithio)propionate, a new heterobifunctional reagent. Biochem. J., 173:727-737, 1978.
16. Kensil, C.R., Patel, U., Lennick, M., and Marciani, D. Separation and characterization of saponins with adjuvant activity from Quillaja saponaria molina cortex. J. Immunol., 146:431-437, 1991.
17. Livingston, P.O., Ritter, G., and Calves, M.J. Antibody response after immunization with the gangliosides GM1, GM2, GM3, GD2, and GD3 in the mouse. Cancer Immunol. Immunother., 29:179-184, 1989.
18. Zhang, S., Helling, F., Lloyd, K. O., and Livingston, P. O. Increased tumor cell reactivity and complement-dependent cytotoxicity with mixtures of monoclonal antibodies against different gangliosides. Cancer Immunol. Immunother., 40:88-94, 1995.
19. James S. P. Measurement of proliferative responses of cultured lymphocytes. In Current Protocols in Immunology (J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds.) pp. 7.10.1-7.10.10. John Wiley & Sons, Inc. New York, 1994.
20. Luo, Y, and Dorf, M. E. Delayed-type hypersensitivity. In Current Protocols in Immunology (J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds.) pp. 4.5.1-4.5.5. John Wiley & Sons, Inc. New York,

1994.

21. Wunderlich, J. and Shearer, G. Induction and measurement of cytotoxic T lymphocyte activity. In Current Protocols in Immunology (J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds.) pp. 3.11.1-3.11.15. John Wiley & Sons, Inc. New York, 1994.
22. Dohi, T., Nores, G. A., Oguchi, H., Inufusa, H., and Hakomori, S. GD3 lactone as an immunogen associated with melanoma: effect of immunization with GM3 lactone on melanoma growth in vivo. In Gangliosides and Cancer (H. F. Oettgen, eds.) pp 275-281, VCH Verlagsgesellschaft, Weinheim (Germany) and VCH Publishers (New York, U.S.A.), 1988.
23. Rughetti A., Turchi V., Ghetti C. A., Scambia, G., Panici, P. B., Roncucci, G., Mancuso, S., Frati, L., and Nuti, M. Human B-cell immune response to the polymorphic epithelial mucin. Cancer Res., 53:2457-2459, 1993.
24. Jerome, K. R., Barnd, D. L., Bendt, K. M., Boyer, C. M., Taylor-Papadimitriou, J., McKenzie, I. F. C., Bast, R. C. Jr., and Finn, O. J. Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. Cancer Res., 51:2908-2916, 1991.
25. Ioannides, C. G., Fisk, B., Jerome, K. R., Irimura, T., Wharton, J. T., and Finn, O. J. Cytotoxic T cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. J. Immunol., 151:3693-3703, 1993.
26. Apostolopoulos, V., Xing, P-X., and McKenzie, F. C. Murine immune response to cells transfected with human

MUC1: immunization with cellular and synthetic antigens. Cancer Res., 54:5186-5193, 1994.

- 5 27. Ding, L., Lalani, E-N., Reddish, M., Koganty, R., Wong, T., Samuel, J., Yacyshyn, M. B., Meikle, A., Fung, P. Y. S., Taylor-Papadimitriou, J., and Longenecker, B. M. Immunogenicity of synthetic peptides related to the core peptide sequence encoded by the human MUC1 mucin gene: effect of immunization on the growth of murine mammary adenocarcinoma cells transfected with the human MUC1 gene. 10 Cancer Immunol. Immunother., 36:9-17, 1993.
- 15 28. Bu, D., Domenech, N., Lewis, J., Taylor-Paradimitriou, J., and Finn, O. J. Recombinant vaccine mucin vector: in vitro analysis of expression of tumor-associated epitopes for antibody and human cytotoxic T-cell recognition. J. Immunol., 14:127-135, 1993.
- 20 29. Newmann, M. J., Wu, J-Y., Gardner B. H., Munroe, K. J., Leombruno, D., Recchia, J., Kensil, C.R., and Coughlin, R. T. A sponin adjuvant induction of ovalbumen-specific CD8+ cytotoxic T-lymphocyte responses. J. Immunol., 148:2357-2360, 1992.
- 25 30. Finn, O. J. Immunity to epithelial tumors and mucin-based vaccine design. Proc. Am. Assoc. Cancer Res., 36:675, 1995.
- 30 31. Engelhard, V. H. Structure of peptides associated with class I and Class II MHC molecules. Annu. Rev. Immunol., 12:181-207, 1994.



SECOND SERIES OF EXPERIMENTS

The mucin MUC-1 is expressed on breast carcinomas in an under glycosylated configuration and is therefore a target for immune recognition. A 30 amino acid (aa) sequence of MUC-1 has been synthesized and in order to augment its immunogenicity, has been covalently linked to KLH and mixed with the immune adjuvant QS21. Eligibility criteria include: patients with stage 4 NED (no evidence of disease), elevated CEA or CA15-3 levels and NED, or initially unresectable stage 3 post adjuvant therapy (tx). Five vaccines, each containing 100 mcg of MUC-1 peptide, were given on weeks 1, 2, 3, 7, 19. Thus far five pts are on study, although only 5 patients have received  $\geq 3$  vaccinations. Local erythema and induration at the injection site and mild flu-like symptoms most prominent after vaccination # 2 were observed in all pts. Patient sera were analyzed by ELISA for IgG and IgM antibodies against purified MUC-1 and by an immune adherence rosetting assay against MUC-1 positive or negative cell lines. IgM/IgG titers by ELISA for the first five patients were:

Week#	0	3	8	20
Pt#1	0/0	10240/40	2560/160	320/1280
Pt#2	0/0	180/640	2560/2560	
Pt#3	0/0	2560/320	2560/2560	
Pt#4	0/0	1280/80	2560/2560	
Pt#5	0/0	320/320	640/320	

For Pt# 1 to Pt# 4 immune adherence assays measuring IgM reactivity against MCF-7 cells gave a titer of 0 pre therapy which have risen to a titer of 160 by week 9. The fifth patient (Pt# 5) started with a titer of 80 pre therapy and increased to 160 by week 9. The MUC-1 mucin is strongly immunogenic in breast cancer patients when presented in a vaccine containing KLH and QS 21.

THIRD SERIES OF EXPERIMENTS

Stage IV no evidence of disease (NED) breast cancer patients (BCPts) or earlier stage BCPTs except for rising CEA or BR2729 levels are at high risk for overt recurrence and might benefit from immunotherapy. Mucin MUC-1, found on most breast carcinomas, is a potential target. A synthetic 30 amino acid (aa) sequence of MUC-1 has been conjugated with KLH and mixed with the immune adjuvant QS-21 to increase immune recognition. Nine patients (ages 43-61) have been vaccinated: eight stage IV NED, one stage II with increased CEA level and NED, all but one stage IV NED patient on hormonal treatment. All patients received five doses of 100 mcg MUC-1 s.c. given on weeks 1, 2, 3, 7, and 19. All patients had transient grade 2 local toxicity at the vaccine site and most had grade 1-2 flu-like symptoms. All patients remain NED (median follow up 55 weeks) although one patient had a chest wall recurrence which was excised. For all patients, the range of IgM and IgG reciprocal titers against purified MUC-1 by ELISA are:

Week #	IgM Titers	IgG Titers
0	0-160	0-10
3	10-20,480	0-320
5	1280-20,480	40-20,480
13	10-20,480	160-2560
21	320-30,480	640-10,240

Five patients maintain IgG titers (range 320-1280) between six-twelve months following the last vaccine. Analysis of IgG subclass in eight patients reveal predominantly IgG1 and IgG3. Immune adherence rosetting against MCF-7 cell lines revealed an increase in IgM titers in 6/7 patients. Inhibition assays demonstrate that all sera react exclusively with the APDTRPA determinant of MUC-1. No evidence for augmentation of T cell immunity was found.

This MUC-1 vaccine is immunogenic in breast cancer patients who are NED.

#### FOURTH SERIES OF EXPERIMENTS

5 The mucin MUC-1 is expressed on breast cancers in an underglycosylated form compared to normal tissues and is therefore a potential target for cancer immunotherapy. MUC-1 contains multiple tandem repeats of the 20 amino acid  
10 peptide (VTSAPDTRPAPGSTAPPAHG). The APDTR epitope is particularly immunogenic since it is recognized by a variety of murine monoclonal antibodies and immune sera, and by some sera and cytotoxic T cells from unimmunized patients with epithelial cancers. A 30 amino acid peptide  
15 VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA was prepared with cysteine at the N-terminal end for chemical conjugated to keyhole limpet hemocyanin (KLH). Six breast cancer patients immunized in the adjuvant setting with this conjugate plus the immunological adjuvant QS-21 have all produced high  
20 titer (by ELISA) IgG and IgM antibodies against the 30 amino acid MUC-1 peptide. A series of smaller peptides were prepared to determine the epitopes recognized by these immune sera in inhibition assays. Only peptides containing APDTRPA were able to inhibit ELISA reactivity for the full  
25 30 amino acid peptide. No sera were inhibited by APDTR, APDTRP, PDTRPA or any other peptides that did not contain the full APDTRPA epitope. Remarkably, sera from all six patients recognized this same epitope and only this epitope. Reactivity was greatest, however, when the  
30 APDTRPA was at the C-terminal end of the peptide, raising the possibility that it is recognized preferentially because it was terminal in the immunogen as well. An additional group of patients are planned to be immunized with a conjugate vaccine containing MUC-1 peptide with  
35 other amino acids at the C-terminal end.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Livingston, Philip O  
Zhang, Shengle
- 10 (ii) TITLE OF INVENTION: Conjugated Mucin Peptide Vaccines
- (iii) NUMBER OF SEQUENCES: 7
- 15 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Cooper & Dunham LLP  
(B) STREET: 1185 Avenue of the Americas  
(C) CITY: New York  
(D) STATE: NY  
20 (E) COUNTRY: U.S.A.  
(F) ZIP: 10036
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
25 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
30 (B) FILING DATE:  
(C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: White, John P  
35 (B) REGISTRATION NUMBER: 28,678  
(C) REFERENCE/DOCKET NUMBER: 50397-A-PCT/JPW/AKC
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 212-278-0400  
40 (B) TELEFAX: 212-391-0525
- (2) INFORMATION FOR SEQ ID NO:1:
- 45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
50 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- 55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
Gly Val Thr Ser Ala Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala  
1 5 10 15
- 60 (2) INFORMATION FOR SEQ ID NO:2:  
(i) SEQUENCE CHARACTERISTICS:

- 35 -

(A) LENGTH: 21 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His  
1 5 10 15  
Gly Val Thr Ser Cys  
20

20 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr Arg Pro  
1 5 10 15  
Ala Pro Gly Ser Cys  
20

40 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31 amino acids  
(B) TYPE: amino acid  
45 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His  
1 5 10 15  
Gly Val Thr Ser Ala Pro Asp Thr Arg Pro Ala Pro Gly Ser Cys  
20 25 30

60 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 36 -

(A) LENGTH: 31 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys Val Thr Ser Ala Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala  
1 5 10 15

15

Pro Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr Arg Pro Ala  
20 25 30

20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 51 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Cys Val Thr Ser Ala Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala  
1 5 10 15

35

Pro Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr Arg Pro Ala Pro  
20 25 30

40

Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr  
35 40 45

Arg Pro Ala  
50

45

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: DNA (genomic)

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala Pro Pro Ala His Gly  
1 5 10 15

60

Val Thr Ser Ala  
20

**What is claimed is:**

1. A vaccine capable of producing an immune response which recognizes a mucin, comprising an amount of mucin peptide conjugated to an immunogenic protein effective to stimulate or enhance immune response in the subject, an effective amount of an adjuvant and a pharmaceutically acceptable vehicle.
2. The vaccine of claim 1, wherein the subject is a human.
3. The vaccine of claim 1, wherein the immunogenic protein is Keyhole Limpet Hemocyanin or a derivative of Keyhole Limpet Hemocyanin.
4. The vaccine of claim 1, wherein the mucin is MUC1.
5. The vaccine of claim 1, wherein the mucin is selected from a group consisting of MUC2, MUC3, MUC4 and MUC5.
6. The vaccine of claim 4, wherein the mucin peptide ranges from ten amino acids to three hundred amino acids in length.
7. The vaccine of claim 4, wherein the mucin peptide is selected from the group consisting of APDTRPAPGSTAPPAHGVTS, TAPPAHGVTSAPDTRPAPGS, APDTRPAPGSTAPPAHGVTSAPDTRPAPGS, VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA, and (VTSAPDTRPAPGSTAPPAH)<sub>2</sub>VTSAPDTRPA.
8. The vaccine of claim 7, wherein the mucin peptide is VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA.
9. The vaccine of claim 6, wherein the effective



amount of conjugated mucin peptide is an amount between about 1  $\mu$ g and about 1mg.

- 5           10.       The vaccine of claim 1, wherein the adjuvant is QS-21.
11.       The vaccine of claim 10, wherein the effective amount of QS-21 is an amount between about 10 $\mu$ g and about 200 $\mu$ g.
- 10           12.       The vaccine of claim 11, wherein the effective amount of QS-21 is about 100  $\mu$ g.
13.       The vaccine of claim 1, wherein the subject is afflicted with cancer and the immune response produced in the subject upon administration of the vaccine effectively treats the cancer.
- 15           14.       The vaccine of claim 1, wherein the subject is susceptible to cancer and the immune response produced in the subject upon administration of the vaccine effectively prevents the cancer.
- 20           15.       The vaccine of claim 14, wherein cells of the cancer have the mucin on their surface.
- 25           16.       The vaccine of claim 14, wherein the cancer is a breast cancer, prostate cancer, colon cancer, lung or pancreas cancer.
- 30           17.       A method for stimulating or enhancing in a subject production of an immune response which recognizes the mucin comprising administering to the subject an effective dose of the vaccine of claim 1.
- 35           18.       A method for treating cancer in a subject afflicted with cancer comprising administering to the subject

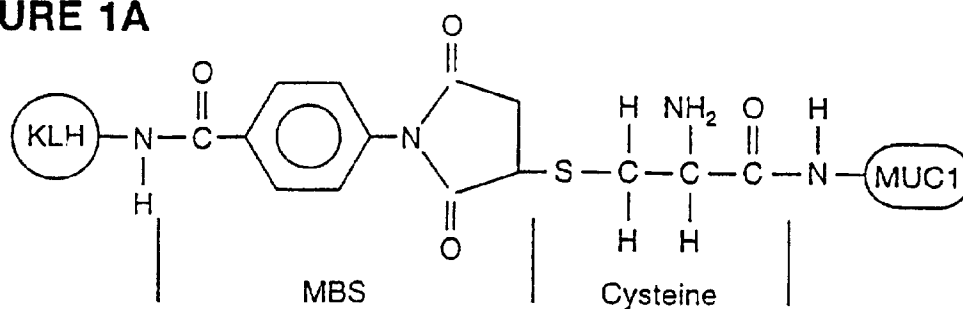
an effective dose of the vaccine of claim 1.

- 5 19. A method for preventing cancer in a subject susceptible to cancer comprising administering to the subject an effective dose of the vaccine of claim 1.
- 10 20. A method for preventing the recurrence of a cancer in a subject susceptible to cancer comprising administering to the subject an effective dose of the vaccine of claim 1.
- 15 21. The method of claim 17, 18, 19 or 20 wherein the immunogenic protein is Keyhole Limpet Hemocyanin or a derivative of Keyhole Limpet Hemocyanin.
22. The method of claim 17, 18, 19 or 20, wherein the adjuvant is QS-21.
- 20 23. The method of claim 17, 18, 19 or 20, wherein the mucin is MUC1.
- 25 24. The method of claim 17, 18, 19 or 20, wherein the mucin is selected from a group consisting of MUC2, MUC3, MUC4 and MUC5.
- 30 25. The method of claim 23, wherein the mucin peptide ranges from ten amino acids to three hundred amino acids in length.
- 35 26. The method of claim 25, wherein the mucin peptide is selected from the group consisting of APDTRPAPGSTAPPAHGVTS, TAPPAHGVTSAPDTRPAPGS, APDTRPAPGSTAPPAHGVTSAPDTRPAPGS, VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA, and (VTSAPDTRPAPGSTAPPAHG)<sub>2</sub>VTSAPDTRPA.

27. The method of claim 26, wherein the mucin peptide is VTSAPDTRPAPGSTAPPAHGVTSAPDTRPA.
- 5 28. The method of claim 23, wherein the effective amount of conjugated mucin peptide is an amount between about 1  $\mu\text{g}$  and about 1mg.
29. The method of claim 25, wherein the adjuvant is QS-21.
- 10 30. The method of claim 29, wherein the effective amount of QS-21 is an amount between about 10 $\mu\text{g}$  and about 200 $\mu\text{g}$ .
- 15 31. The method of claim 30, wherein the effective amount of QS-21 is about 100  $\mu\text{g}$ .
32. The method of claim 18, 19 or 20, wherein cells of the cancer have the mucin on their surface.
- 20 33. The method of claim 18, 19 or 20, wherein the cancer is a breast cancer, prostate cancer, colon cancer, lung or pancreas cancer.

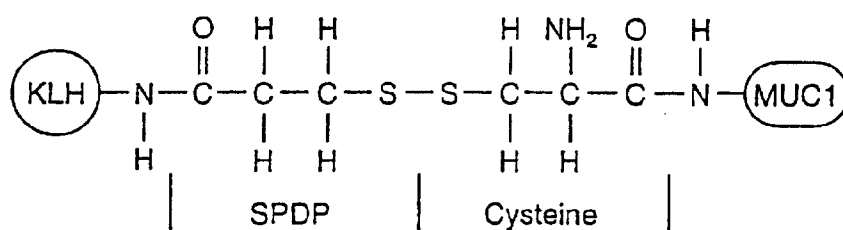
1/1

FIGURE 1A



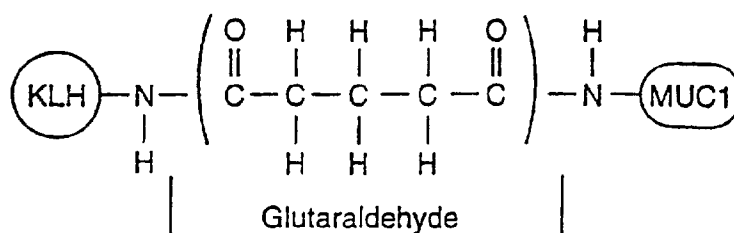
MBS Linker

FIGURE 1B



SPDP Linker

FIGURE 1C



Glutaraldehyde Linker

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/04493

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C07K 7/04; A61K 38/17

US CL :424/184.1, 185.1, 193.1; 514/2; 530/300, 324, 326

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 185.1, 193.1; 514/2; 530/300, 324, 326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, BIOSIS, EMBASE

search terms: mucin, vaccine, cancer

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	DING et al. Immunogenecity of Synthetic Peptides Related to the Core Peptide Sequence Encoded by the Human MUC1 Mucin Gene: Effect of Immunization on the Growth of Murine Mammary Adenocarcinoma Cells Transfected with the Human MUC1 Gene. Cancer Immunolo. Immunother. 1993, Vol 36, pages 9-17, see entire document.	1, 3-4, 6, 9, 13-15, 17-19, 21, 23, 25, 28, 32 ----- 2, 5, 10-12, 16, 20, 22, 24, 29-31, 33

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 22 MAY 1997	Date of mailing of the international search report 21 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer NANCY A. JOHNSON
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/04493

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	APOSTOLOPOULUS et al. Murine Immune Response to Cells Transfected with Human MUC1: Immunization with Cellular and Synthetic Antigens. Cancer Research. 01 October 1994, Vol. 54, pages 5186-5193, see entire document.	1,2,4,6,9,13-20,23,25,28,32-33 ----- 3,5,10-12,21-22,24,29-31
Y	FINN. Immunity to Epithelial Tumors and Mucin-Based Vaccine Design. Proceedings of the American Association for Cancer Research. March 1995, Vol. 36, page 675, see entire document.	1-6, 9-25, 28-33
Y	HOLLINGSWORTH et al. Expression of MUC1, MUC2, MUC3 and MUC4 MUCIN mRNAs in Human Pancreatic and Intestinal Tumor Cell Lines. Int. J. Cancer. 1994, Vol. 57, pages 198-203. see entire document.	5, 24
Y	US 5,455,034 A (NAGARAJA and CHENGAPPA) 03 October 1995, column 14, lines 26-32.	10-12, 22, 29-31
X ---- Y	US 5,229,289 A (KJELDSSEN et al.) 20 July 1993, see entire document.	1-2, 9, 13-20, 32, 33 ----- 3-6, 10-12, 21-25, 28-31

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